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Properties of the newly isolated extracellular thermo-alkali-stable laccase from thermophilic actinomycetes, *Thermobifida fusca* and its application in dye intermediates oxidation

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Abstract

Laccases are diphenol oxidases that have numerous applications to biotechnological processes. In this study, the laccase was produced from the thermophilic actinomycetes, *Thermobifida fusca* BCRC 19214. After 36 h of fermentation in a 5-liter fermentor, the culture broth accumulated 4.96 U/ml laccase activity. The laccase was purified 4.64-fold as measured by specific activity from crude culture filtrate by ultrafiltration concentration, Q-Sepharose FF and Sephacryl™ S-200 column chromatography. The overall yield of the purified enzyme was 7.49%. The molecular mass of purified enzyme as estimated by SDS-PAGE and by gel filtration on Sephacryl™ S-200 was found to be 73.3 kDa and 24.7 kDa, respectively, indicating that the laccase from *T. fusca* BCRC 19214 is a trimer. The internal amino acid sequences of the purified laccase, as determined by LC-MS/MS, had high homology with a superoxide dismutase from *T. fusca* YX. Approximately 95% of the original activity remained after treatment at 50°C for 3 h. and approximately 75% of the original activity remained after treatment at pH 10.0 for 24 h. This laccase could oxidize dye intermediates, especially 2,6-dimethylphenylalanine and *p*-aminophenol, to produce coloring. This is the first report on laccase properties from thermophilic actinomycetes. These properties suggest that this newly isolated laccase has potential for specific industrial applications.

Keywords: Laccase; Dye intermediate; Thermophilic; *Thermobifida fusca*; LC-MS/MS

Introduction

Laccases (E.C. 1.10.3.2) are well-known enzymes that were first isolated from the lacquer tree, *Rhus vernicifera*. They have received increasing attention in recent decades due to their ability to oxidize both phenolic and nonphenolic lignin-related compounds and highly recalcitrant environmental pollutants, which makes them very useful for applications related to biotechnological processes (Mukhopadhyay et al., 2013; Couto and Herrera, 2006; Albino et al., 2004). Laccases are members of the multi-copper oxidase family of enzymes that contain four copper atoms in their functional units. Laccases have been isolated from many plants, fungi and bacteria. Most known laccases are of fungal origin, and

they participate in a variety of physiological functions, such as stress defense and lignin degradation (Baldrian, 2006; Giardina et al. 2010; Kües and Rühl 2011).

One application for laccase is hair coloring. Oxidation-based hair color, which has dominated the hair color market, consists of dye intermediates and an oxidizing agent (Saito et al., 2012). In a typical hair color product, the dye intermediates are *p*-diamines and *p*-aminophenols, and hydrogen peroxide is used as the oxidant in the dyeing process. After mixing, they form chromatic indo dyes at the time of use. However, side reactions with hair proteins commonly occur simultaneously because of the severe reactions conditions, resulting in hair damage (Saito et al., 2012). The commercial hydrogen peroxide oxidative-type hair dyeing formulations are mutagenic. Some hair dyeing components become strongly mutagenic after oxidation by hydrogen peroxide (Ames et al., 1975). Laccase-based hair dyes are less irritating and easier to handle than

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current hair dyes, as laccases replaced hydrogen peroxide as the oxidizing agent in the dyeing formula (Couto and Herrera, 2006).

Streptomycetes are widespread soil actinomycetes that play important roles in the decomposition of biopolymers such as lignin, cellulose, hemicellulose, chitin, keratin and pectin (Locci, 1989). Although most laccases are found in mesophilic microorganisms, thermophilic microorganisms are considered to be good sources of thermostable and novel enzymes with potential industrial importance. However, little has been reported on laccase production by thermophilic actinomycetes. To produce enzymes for the development of enzymatic degradation of renewable lignocellulose, we isolated 70 potent extracellular lignocellulolytic enzyme-producing thermophilic actinomycetes from compost soils collected in Taiwan (Yang et al., 2009). Of the 70 strains of thermophilic actinomycetes, strain No. 10-1 had the best laccase activity. According to its biological properties and 16S rDNA similarity, this newly isolated strain was identified as *Thermobifida fusca* and deposited in the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) with stock number BCRC 19214 (Chen et al., 2013). Database mining of the complete genome sequence of *T. fusca* YX, which was accessible in 2007 (Lykidis et al. 2007), did not find genes that encode fungal laccase-like proteins. There are no reports on laccase production and properties by *T. fusca* before.

The coloring reactions are usually carried out at alkaline pH because the hair swells and the penetration of dyes is enhanced. Although many laccases have been isolated, few studies have reported laccases with high activity under neutral or alkaline conditions (Gouka et al., 2001; Heinzkill

et al., 1998; Sulistyanyndyah et al., 2004). The purpose of this study was to produce thermo-alkali-stable laccase from thermophilic actinomycetes. The enzyme properties and dye intermediates oxidation application of the laccase were also investigated. The results of this investigation have implications for cosmetics and human health.

Materials and methods

Microorganisms

T. fusca BCRC 19214, cultivated routinely in CYC medium (Czapek-Dox powder 33 g/L, yeast extract 2 g/L, casamino acids 6 g/L, pH 7.2) at 50°C, was studied in this study. This strain was deposited in the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) with stock number.

Materials

Sugarcane bagasse, corncob, and pine sawdust were collected from the local market and washed extensively with running water until the residual soluble sugar was removed. Then, they were dried and mixed in a blender. The resulting small pieces, passed through a 100 mesh screen, were collected and used in this study. Czapek-dox powder, yeast extract, casamino acids, and agar were obtained from BD (Sparks, MD, USA). Q-Sepharose FF and Sephacryl™ S-200 were supplied by GE Healthcare (Little Chalfont, UK). The protein assay kit and SDS-PAGE molecular weight standards were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Dye intermediates, inorganic salts and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

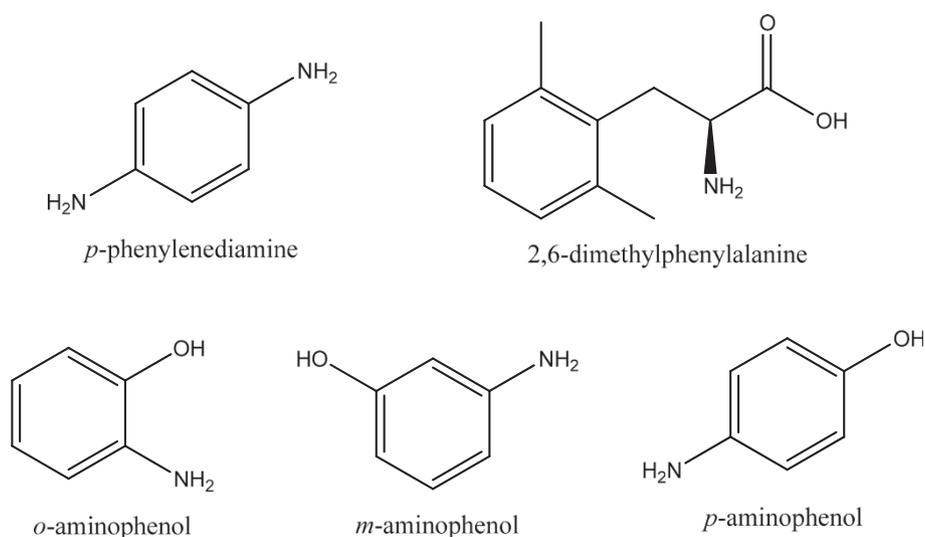
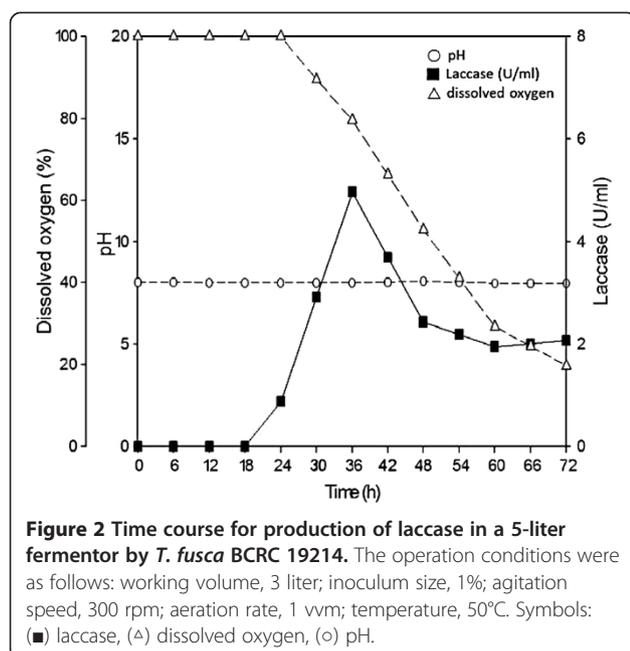


Figure 1 Chemical structure of dye intermediates.



Cultivation in a fermentor

The laccase-producing strain was cultivated in a 5-liter fermentor (Biostat B, B. Braun, Melsungen, Germany). A 500-ml Hinton flask containing 100 ml of bagasse medium was inoculated with the strain cultured at 50°C and shaken (150 rpm) for 48 h; this was used as the seed culture. The 5-liter fermentor was loaded with 3 liters of bagasse medium. Cultivation was performed at 50°C, 1.0 v.v.m. aeration and 300 rpm agitation.

Enzyme purification

All purification procedures were performed at 4°C in 20 mM phosphate buffer (pH 8.0) unless otherwise stated. After 36 h cultivation of the laccase-producing strain in a 5-liter fermentor, the fermentation broth was centrifuged at $10,000 \times g$ for 30 min to remove the cells. The supernatant was concentrated by ultrafiltration (Pellicon XL, Biomax 10 K, Millipore). The concentrated solution was then applied to a Q-Sepharose FF anion-exchange column (1.13 cm \times 5 cm) that was pre-equilibrated with phosphate buffer. After washing with the same buffer to remove inactive protein, the enzyme was eluted with a linear gradient of the buffer containing NaCl from 0.0 M to 1.0 M (flow rate: 180 ml/h). Enzyme activity was detected within the range 0.4 M to 0.6 M NaCl. The main active fractions were applied to a Sephacryl™ S-200 column (1.6 \times 90 cm) that was previously equilibrated with 20 mM phosphate buffer. Proteins were eluted at a flow rate of 30 ml/h. The eluted

enzymatically active fractions were pooled and used as the purified enzyme.

Polyacrylamide gel electrophoresis

SDS-PAGE was performed using 10% gels to determine the molecular weight of the purified protein and the purity of each purification step. Coomassie Brilliant Blue R-250 was used for protein staining. LMW-SDS Maker (GE Healthcare, Little Chalfont, UK) was used as the standard. Native-10% PAGE was performed according to standard SDS-PAGE procedures, but the gels did not contain SDS. The samples were not heated, and no SDS or β -mercaptoethanol was added. The electrophoresis was performed at 150 V for 1 h at 4°C. After electrophoresis, the native-PAGE was washed in 20 mM phosphate buffer (pH 8.0) at 4°C and 50 rpm for 90 min, with buffer changes every 30 min. The native-PAGE was soaked in 20 mM 2,6-DMP (dissolved in 20 mM phosphate buffer) and then incubated at 50°C until bands began to appear.

Internal amino acid sequence of laccase by LC-MS/MS

The laccase internal amino acid sequence was performed by in-gel digestion of the protein and sequencing of the different peptides by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an Applied Biosystems QStar LC-MS/MS spectrometer (Life Technologies Corp., Carlsbad, USA) as described previously (Chen et al., 2013). The analysis was performed at the Biotechnology center, NCHU (National Chung Hsing University). The mass spectrometry information was analyzed using Mascot software (Matrix Science Ltd., London, UK) and the NCBI database. The peptide mass accuracy was ± 0.5 Da for Mascot analysis. The resulting amino acids were matched to the NCBI database.

Laccase activity

Unless otherwise indicated, the standard laccase activity assay was carried out at 50°C for 15 min, using 20 mM 2,6-dimethoxyphenol (2,6-DMP) as substrate in 20 mM phosphate buffer (pH 8.0). 2,6-DMP oxidation was monitored by the increase in absorbance at 470 nm ($\epsilon_{470} = 35645 \text{ M}^{-1} \text{ cm}^{-1}$). One unit was defined as the activity required to oxidize 1 nmol of the substrate per minute under the indicated reaction conditions (Chen et al., 2013).

Oxidative activities for dye intermediates

The dye intermediates used in this study are shown in Figure 1. The oxidation activities using *p*-phenylenediamine, 2,6-dimethylphenylalanine, and aminophenols were determined in a DMSO solution (final concentration was 0.5%). Oxidation was spectroscopically monitored in phosphate buffer (pH 8.0). The final concentrations of the

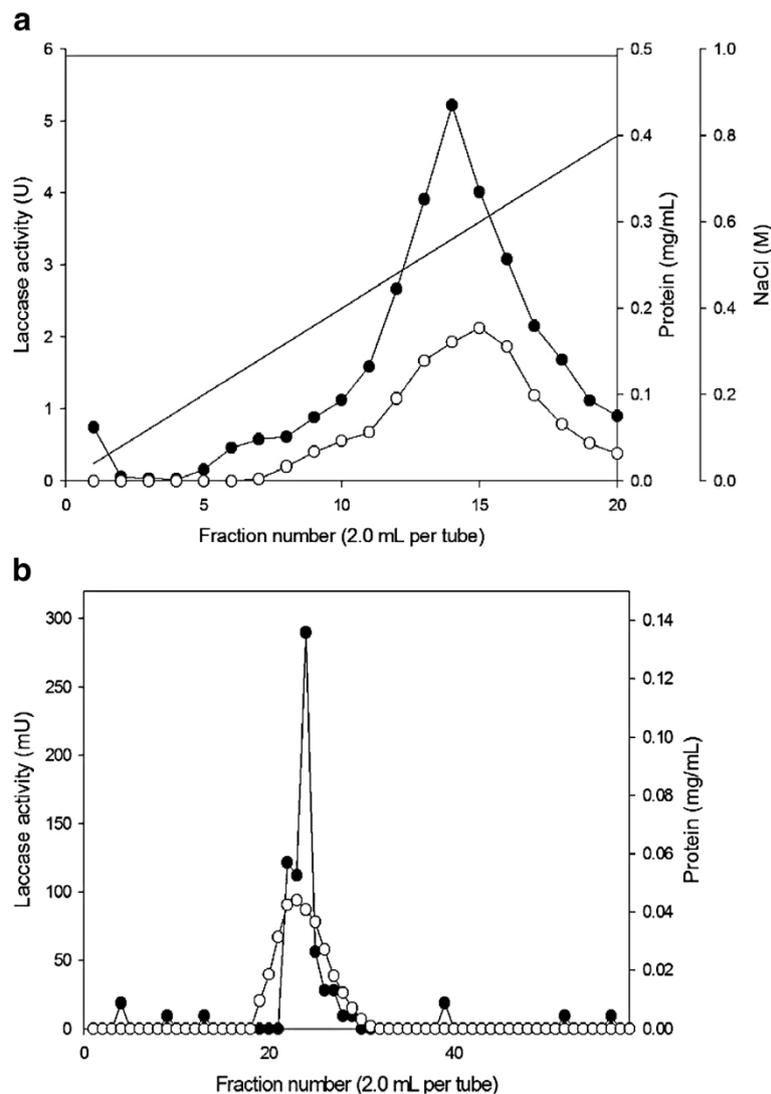


Figure 3 Purification of laccase by column chromatography. (a) Q-Sepharose FF chromatography. (●) laccase activity; (○) Protein (mg/ml); (---) NaCl gradient. Column: 1.13 × 5 cm; flow rate: 180 ml/h. (b) Sephacryl™ S-200 chromatography. (●) laccase activity; (○) Protein (mg/ml); (---) NaCl gradient. Column: 1.6 × 90 cm; flow rate: 30 ml/h.

substrates and the buffer were 20 mM. The oxidation was monitored at 470 nm.

Statistical analysis

All analytic measurements were performed at least 3 times. The data are expressed as the mean ± SD.

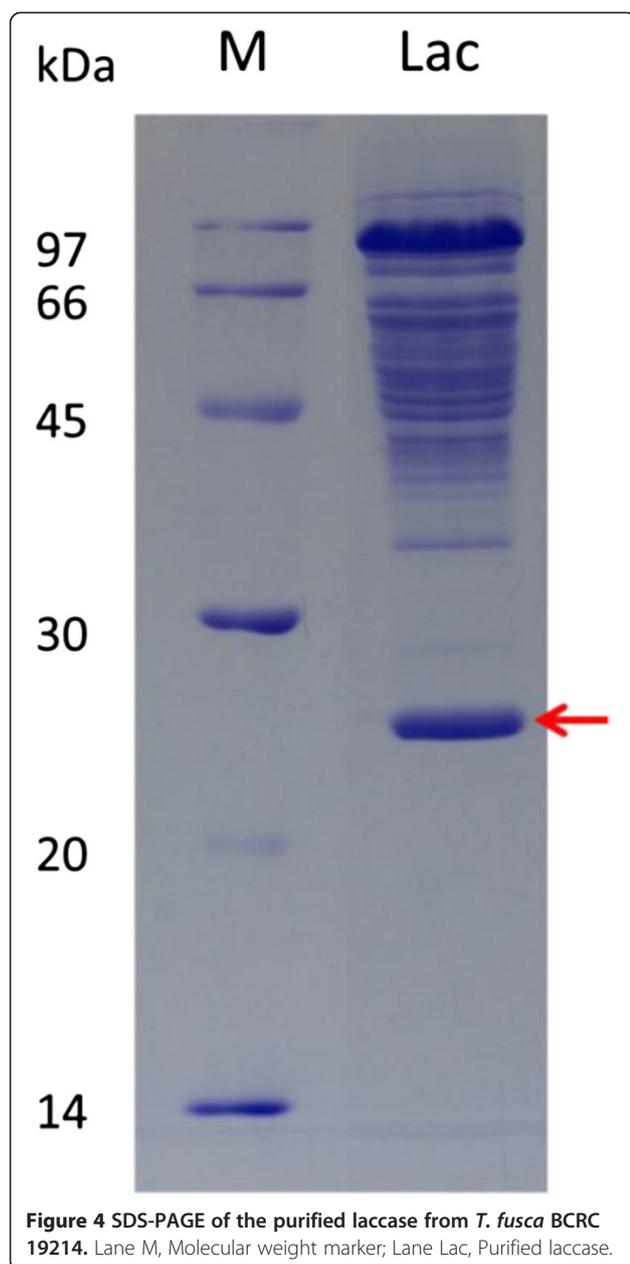
Results

Production of the laccase in fermentor

Bagasse, corncob and pine sawdust were used as carbon sources to produce the laccase from *T. fusca* BCRC 19214. The highest extracellular laccase activity was observed when *T. fusca* BCRC 19214 was grown in a mineral medium containing bagasse as the carbon source.

Table 1 Summary of the purification of laccase from *T. fusca* BCRC 19214

Step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude culture filtrate	760.00	12.31	49.04	3.98	1.00	100.00
Ultrafiltration	20.00	7.94	39.09	11.27	2.83	79.71
Q-Sepharose FF	16.00	2.36	30.75	13.05	3.28	62.71
Sephacryl™ S-200	6.00	0.20	3.68	18.50	4.64	7.49



Next, the culture conditions for the production of laccase were investigated in a 5-liter fermentor. Typical enzyme levels produced by *T. fusca* BCRC 19214 in a 5-liter fermentor are shown in Figure 2. The rapid consumption of oxygen paralleled an increase in laccase activity during the growth phase of the culture. The appearance of laccase activity in the culture broth became significant after 24 h of cultivation. Maximum laccase activity was approximately 4.96 U/ml in the culture broth after 36 h of cultivation, and the laccase activity decreased after further cultivation time.

Purification of laccase from *T. fusca* BCRC19214

The purification of laccase was performed as described in the Materials and Methods section. Gel-filtration chromatography of Q-Sepharose FF is shown in Figure 3a. The major esterase activity fraction was eluted and applied to a DEAE-Sepharose CL-6B column (Figure 3b). The enzymatically active fractions were eluted, pooled and used as the purified enzyme. The results of the total purification are summarized in Table 1. The purified enzyme obtained exhibited 7.49% of the total initial activity and there was a 4.64-fold increase in its specific activity compared with the crude culture filtrate.

Properties of laccase from *T. fusca* BCRC19214

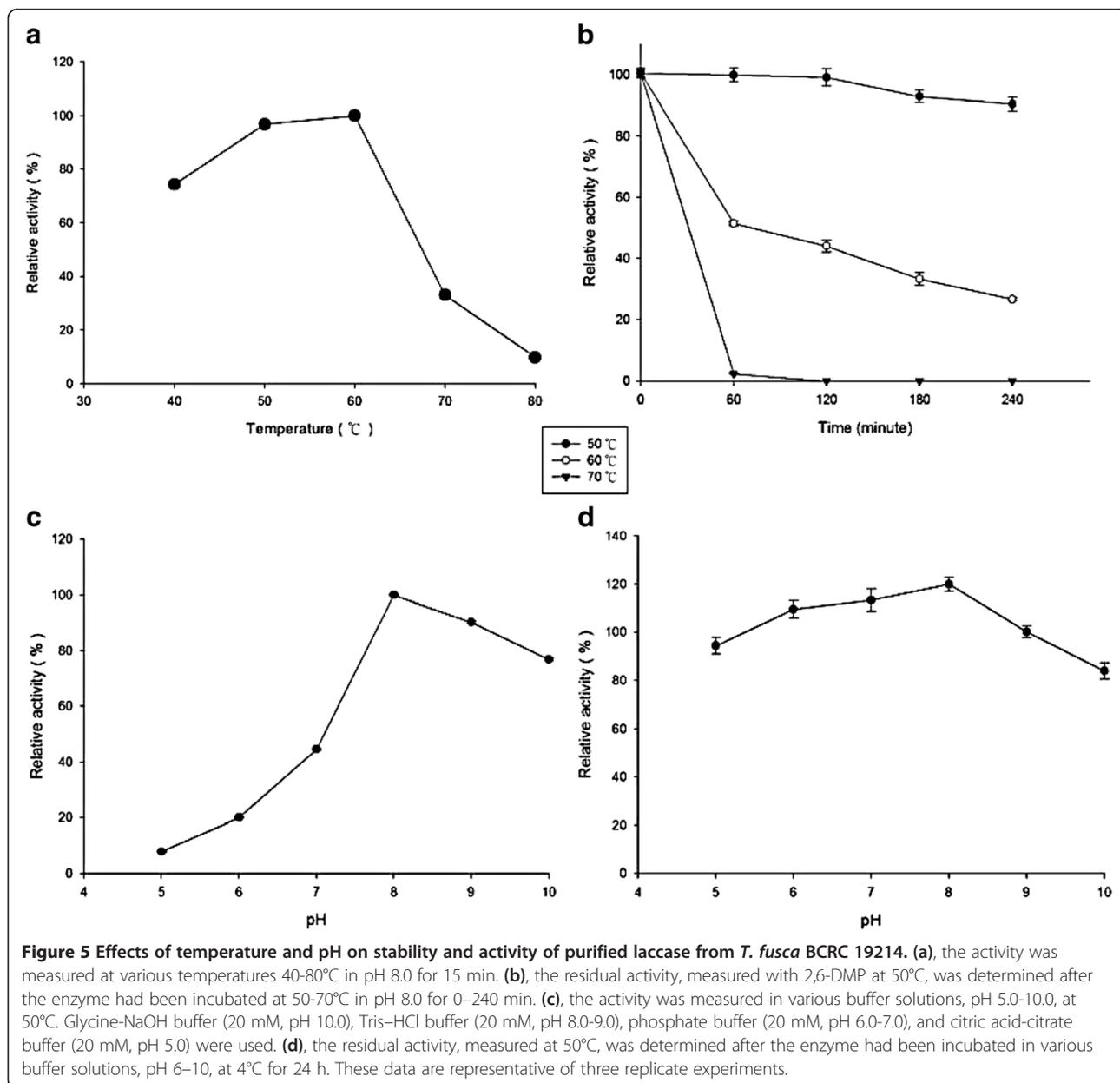
As shown in Figure 4, the purified enzyme showed an apparent single protein band on SDS-PAGE (10% gel). The subunit of the single protein band was estimated to be 24.7 kDa from its mobility relative to those of standard proteins on SDS-PAGE. The molecular mass of the purified enzyme was estimated to be 73.3 kDa by Sephacryl™ S-200 gel filtration, indicating that the purified enzyme is a trimer.

Activity assays were performed using 2,6-DMP as substrate to identify the pH and temperature properties. The optimal pH and temperature of the purified enzyme were 8.0 and 60°C, respectively (Figure 5). The purified enzyme was stable over a pH range of 5.0-10.0 at 4°C for 24 h. Approximately 95% of the original activity remained after treatment at 50°C for 3 h. Approximately 75% of the original activity remained at pH 10.0 for 24 h.

Among the various metal salts and chemical reagents tested, it was found that the purified enzyme activity was partially inhibited by 1 mM Hg²⁺ (Table 2). The presence of β-mercaptoethanol and EDTA significantly reduced the enzyme activity. The enzyme activity partially inhibited by sodium dodecyl sulfate (SDS) and sodium azide (Table 3). The effects of organic solvent on the activity of purified laccase were given in Table 4. With 20% water miscible solvent like methanol, ethanol, isopropanol, acetone, and dimethyl sulfoxide (DMSO) in the reaction system, the activity dropped to 20-74%. Acetonitrile and dimethylformamide (DMF) increased the activities to 110-118%.

Internal amino acid sequence of the laccase by LC-MS/MS

The internal sequences of the purified laccase were determined by digestion with trypsin and sequence analysis using LC-MS/MS. Three sequences (GANDALEQLA EAR, AHFSAAATGIQGSWAILAWDILGQR, and AF WNVVNWADVAK) were detected. Comparisons were then made to all protein sequence in the NCBI database. The results of a BLAST search indicated that the internal sequences had high homology with a superoxide dismutase



(SOD) proteins product from *T. fusca* YX (Accession number 289018.1). We named the protein as Tfu-lac.

Dye oxidation properties

As shown in Table 5, the laccase showed oxidation activity for 5 dye intermediates, including *p*-phenylenediamine, 2,6-dimethylphenylalanine, *o*-aminophenol, *p*-aminophenol and *m*-aminophenol at pH 8.0. The chromogenic reactions of laccase for the dye oxidation widely used in hair color are shown in Figure 6. The laccase exhibited the higher activities toward 2,6-dimethylphenylalanine and *p*-aminophenol. In contrast, the oxidation effect of laccase was less for *m*-aminophenol.

Discussion

Thermophilic actinomycetes are of particular interest because they produce a variety of thermostable enzymes that are involved in the degradation process (Tuncer and Ball, 2002; Yang and Liu, 2004; Yang et al., 2007; Yang and Liu, 2008; Yang et al., 2010; Huang et al. 2010; Huang et al., 2011). *T. fusca* seems to be unique among thermophilic actinomycetes in having a laccase.

Some laccases had been isolated from microorganisms and the properties were summarized in Table 6. Compared with other laccases, the optimum temperature and optimum pH varied from 40°C to 85°C and 3.0 to 9.0, respectively. The laccases from *Bacillus subtilis* and

Table 2 Effect of metal salts on the activity of laccase from *T. fusca* BCRC 19214

Metal salt (1 mM)	Relative activity* (%)
None	100
ZnCl ₂	97 ± 4
HgCl ₂	86 ± 6
CaCl ₂	102 ± 2
FeCl ₂	97 ± 4
MnCl ₂	96 ± 3
MgCl ₂	98 ± 4
NaCl	98 ± 6
KCl	98 ± 3
CoSO ₄	97 ± 4
CuSO ₄	102 ± 5

* The relative activity was measured at 50°C for 15 min, using 20 mM 4-dimethoxybenzylalcohol (veratryl alcohol) as the substrate in 20 mM phosphate buffer (pH 8.0).

Pseudomonas aerophilum had the highest optimal temperature. However, the optimal pH of these two enzymes retained in neutral to acidic (Martins et al., 2002; Fernandes et al., 2010). The laccase from *Streptomyces sviveus* had the wild pH range (5.0-11.0). Approximately 50% of the original activity remained after treatment at 50°C for 226 min. (Gunne and Urlacher, 2012). The laccase purified from *T. fusca* BCRC 19214 had the similar wild pH range with the property from *S. sviveus*. The thermostability of the laccase from *T. fusca* BCRC 19214 was better than the laccase from *S. sviveus*. Therefore, the laccase purified from *T. fusca* BCRC 19214 was an

Table 3 Effect of inhibitors on the activity of laccase from *T. fusca* BCRC 19214

Inhibitor	Concentration (mM)	Relative activity* (%)
None	-	100
SDS	0.1	94 ± 3
	1	73 ± 3
	10	63 ± 6
sodium azide	0.1	87 ± 2
	1	84 ± 4
	10	80 ± 3
β-mercaptoethanol	0.1	86 ± 3
	1	4 ± 2
	10	0 ± 1
EDTA	0.1	24 ± 2
	1	23 ± 2
	10	5 ± 1

* The relative activity was measured at 50°C for 15 min, using 20 mM 2,6-dimethoxyphenol (2,6-DMP) as the substrate in 20 mM phosphate buffer (pH 8.0). SDS, Sodium dodecyl sulfate; EDTA, Ethylenediaminetetraacetic acid.

Table 4 Effect of organic solvents on the activity of laccase from *T. fusca* BCRC 19214

Organic compounds	Concentration (% v/v)	Relative activity* (%)
None	-	100
Methanol	10	59 ± 3
	20	34 ± 3
Ethanol	10	66 ± 3
	20	40 ± 4
Isopropanol	10	61 ± 2
	20	36 ± 4
Acetone	10	50 ± 2
	20	20 ± 1
Dimethyl sulfoxide (DMSO)	10	101 ± 1
	20	74 ± 4
Acetonitrile	10	160 ± 6
	20	118 ± 5
Dimethylformamide (DMF)	10	120 ± 4
	20	110 ± 5

* The relative activity was measured at 50°C for 15 min, using 20 mM 2,6-dimethoxyphenol (2,6-DMP) as the substrate in 20 mM phosphate buffer (pH 8.0).

excellent thermo-alkali-stable laccase for hair coloring and further industrial applications.

Addition of 10 mM SDS lead to a reduction of laccase activity to 63%. Addition of 10 mM sodium azide, a well-known laccase inhibitor, led to a decrease of activity by 20%, whereas many laccases are completely inhibited by concentrations in the micromolar range (Gunne and Urlacher, 2012). The relative stability of the laccase from *T. fusca* BCRC 19214 with chemicals allows use of the enzyme in a wide variety of reaction compositions.

The laccase activity was reduced by metal ions of Hg. It was similar with the results from *Streptomyces psammoticus* (Niladevi et al., 2008), *Trametes hirsute* (Couto and Herrera, 2006), and *Paraconiothyrium variable* (Forootanfar et al., 2011). The role of copper in the enhancement of laccase activity has been well

Table 5 Oxidation activities of laccase from *T. fusca* BCRC 19214 for dye intermediates

Substrate	Relative activity (%)			
	Tfu	Flac1*	BOD*	RvL*
<i>p</i> -phenylenediamine	47.3	800.0	29.4	47.4
<i>o</i> -aminophenol	71.6	2,900.0	547.0	274.7
2,6-dimethylphenylalanine	100.0	100.0	100.0	100.0
<i>p</i> -aminophenol	96.0	100.0	11.7	9.0
<i>m</i> -aminophenol	0.4	-	-	-

*Data from Saito et al., 2012.

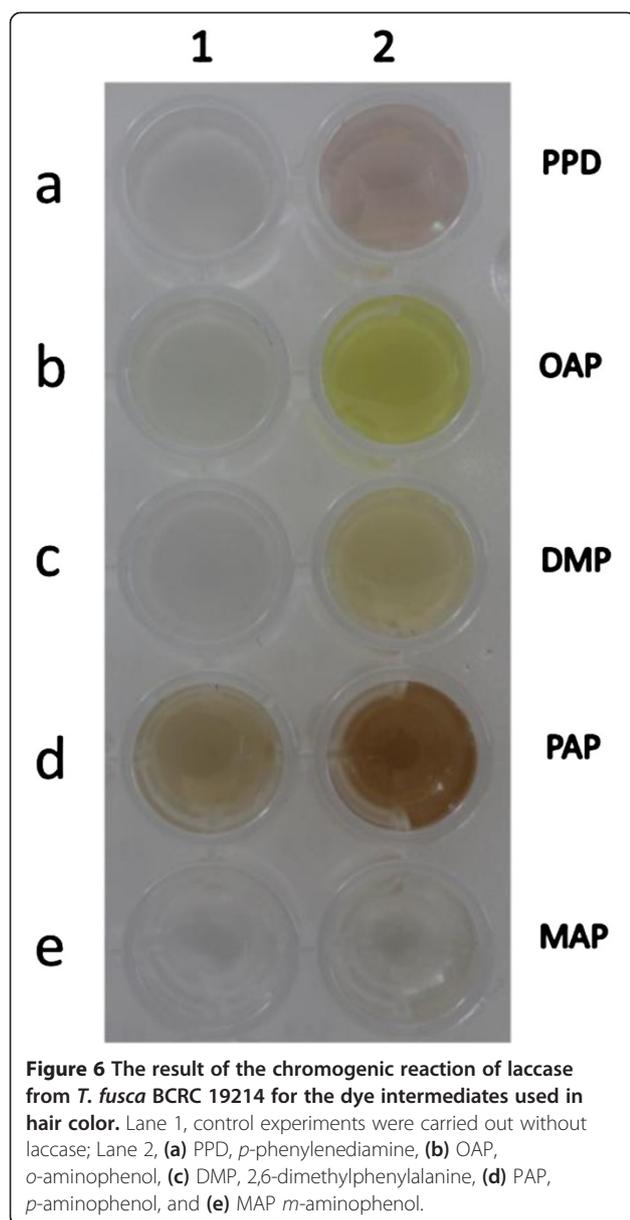


Figure 6 The result of the chromogenic reaction of laccase from *T. fusca* BCRC 19214 for the dye intermediates used in hair color. Lane 1, control experiments were carried out without laccase; Lane 2, (a) PPD, *p*-phenylenediamine, (b) OAP, *o*-aminophenol, (c) DMP, 2,6-dimethylphenylalanine, (d) PAP, *p*-aminophenol, and (e) MAP *m*-aminophenol.

demonstrated in both fungi and bacteria (Givaudan et al., 1993). Similar result has been reported from *Streptomyces cyaneus* (Arias et al., 2003). However, the present result on the effect of Cu on the purified laccase was no enhancement effects.

With 20% water miscible solvent like acetonitrile and DMF in the reaction system, the laccase increased the activities to 110-118%. They were suggested to weaken the hydrophobic interaction and increased the stability of laccase in aqueous solutions (Kovrigin and Potekhin, 2000). With 40% solvent like DMSO, methanol, 2-propanol, acetonitrile, and acetone, the laccase activity from *Streptomyces sviveus* dropped to 20-40% (Günne

Table 6 Biochemical properties of some purified laccase

Source	Optimum temperature	Optimum pH	MW (kDa)	Reference
<i>Trametes versicolor</i>	40	4.0-5.0	66	Minussi et al., 2007
<i>Ganoderma lucidum</i>	60	3	68	Ding et al., 2012
<i>Botrytis cinerea</i>	60	3.5	74	Slomczynski et al., 1995
<i>Chaetomium thermophile</i>	60	6	77	Chefetz et al., 1998
<i>Cladosporium cladosporioides</i>	50	3.5	75.17	Vijaykumar et al., 2011
<i>Schizophyllum commune</i>	40	6	63	Irshad et al., 2011
<i>Pycnoporus cinnabarinus</i>	-	4	81	Eggert et al., 1996
<i>Flammulina velutipes</i>	-	5	28	Saito et al., 2012
<i>Bacillus subtilis</i>	85	4.2	65	Martins et al., 2002
<i>Pseudomonas aerophilum</i>	85	3	49.6	Fernandes et al., 2010
<i>Streptomyces sviveus</i>	-	9	32.5	Günne and Urlacher 2012
<i>Streptomyces psammoticus</i>	45	8.5	43	Niladevi et al., 2008
<i>Paraconiothyrium variabile</i>	50	4.8	84	Forootanfar et al., 2011

and Urlacher, 2012). The effects of organic solvent on the activity of purified laccase from *T. fusca* BCRC 19214 were similar with the results from *S. sviveus*.

This enzyme differed from the sequence of the laccase-like 24.7-kDa copper-containing oxidase Tfu1114 (Genebank number AAZ55152.1) secreted from *T. fusca* (Chen et al., 2013). The results of a BLAST search also indicated that the deduced amino acid sequence of the Tfu-lac protein had higher homologies with five superoxide dismutase (SOD) proteins (YP_007685998.1, YP_062104.1, YP_006642575.1, YP_003678569.1, YP_001625417.1).

Saito et al. investigated the oxidation activities of laccase Flac1 from *Flammulina velutipes* (Saito et al., 2012), bilirubin oxidase (BOD) from *Myrothecium verrucaria* (Guo et al., 1991), and laccase (RvL) from *Rhus vernicifera* (Sulistyaningdyah et al., 2004). All of these enzymes showed the highest activity toward *o*-aminophenol, which differ from the laccase in this study. The laccase from *T. fusca* displayed better activity toward 2,6-dimethylphenylalanine and *p*-aminophenol. This property could be used for developing new dye colors from the intermediates oxidation process.

Competing interests

The authors declare that they have no competing interests.

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